Identifying the Role and Molecular Mechanism of Ubiquitin Ligase Tripartite Motif 27 in Colorectal Cancer Stemness

PhD Synopsis

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1. Background

Colorectal cancer (CRC) is the third most diagnosed cancer with contribution of 10.2% of all cases in the world. It is the third leading cause of cancer related deaths in the world (WHO). Currently, therapeutic options for the treatment of CRC are surgical resection, systemic therapy including chemotherapy, targeted therapy and immunotherapy. Unfortunately even after well-directed curative treatment, patients experience treatment failure that may be associated with the development of multi-drug resistance during or after treatment**.** The multi-drug resistance is mainly contributed by cancer stem cells (CSCs), these are small subpopulation found within the heterogeneous bulk of various tumors (Batlle & Clevers 2017). They are broadly characterized by various properties like asymmetric division, the ability to reconstitute a differentiated tumor upon transplantation, participating in the epithelial-mesenchymal transition (EMT) via induction of genetic programs, and contributing to resistance to traditional chemotherapy regimen due to upregulation of DNA repair components and drug efflux transporters (Dragu et al, 2015). Besides this CSCs are believed to play a critical role in the onset of tumor relapse and metastasis (Wang et al, 2017). CSCs have been reported in a variety of tumor types, although the markers and driver pathways vary among tumor types. However the general stem cell properties and their roles in therapy resistance appear conserved. CSCs often demonstrate the re-expression of embryonic factors including Sox2, Oct4, Nanog, and Klf4. These cells display distinct metabolic profiles from terminally differentiated

tumor cells, and reside in specialized hypoxic microenvironments that contribute to long-term maintenance (Pützer et al, 2017).

According to aforementioned characteristics identifying key molecular networks influencing CSC stemness renders such networks potential targets for cancer therapy. Although transcriptional regulation of stem cell pluripotency and differentiation has been extensively studied (Zakaria et al, 2018), few studies focus on the roles of post-translational modifications in these processes. Ubiquitination is one of the post-translational modifications that play a central role in regulating various biological functions (Seo et al, 2018). This process involves a cascade of enzymatic reactions the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase, with these processes reversed by deubiquitinating enzymes (Rotin et al, 2009). It has been reported that the CSC marker CD133 is target of ubiquitination and is mono-ubiquitinated at lysine 848 (Yang et al, 2018) and ubiquitination of the marker CD166 has been found in head and neck cancer (Xiao et al, 2017). Additionally, tripartite-motif (TRIM) proteins are involved in a variety of cellular functions and biological processes, and recent data revealed their significant role in the acquisition and maintenance of the stem cell phenotype (Jaworska et al, 2020). For example TRIM16 directly regulated the degradation of Gli-1 protein via the ubiquitin-proteasome pathway, which suppressed the CSCs in Breast Cancer (Yao et al, 2016). Moreover, deubiquitylation acts to preserve the cancer stemness, with treatment of deubiquitinase inhibitors resulting in reduction of CSCs and attenuated therapeutic resistance (Qiu et al, 2017). These findings suggest that ubiquitination represents a post-translational modification capable of altering CSC properties. Various evidences from literature demonstrate that TRIM members guarantee the acquisition of stem cell properties and ability to sustain stem-like phenotype by

cancer cells using distinct mechanisms (Hatakeyama et al, 2017). Among these, TRIM27, an E3 ubiquitin ligase acts as positive regulators of stem cell self-renewal property. It has been shown that TRIM27 promotes EMT and activates Phosphatidyinositol-3- Kinase/ Protein Kinase B (PI3/AKT) signaling pathway in CRC cells, leading to the acquisition of a very invasive and metastatic phenotype reflecting the CSC-like phenotype (Zhang et al, 2018). This PI3/AKT is negatively regulated by the tumor suppressor protein Phosphatase and Tensin homolog (PTEN) (Keniry et al, 2008) and is identified as a critical component in this pathway (Joshi et al, 2017). This PTEN/AKT pathway is the common deregulated signaling pathway in most of human cancers (Zhang et al, 2017). Previous reports have demonstrated that the ubiquitination of PTEN is critical for its stability and nuclear localization (Shao et al, 2017). Also the ubiquitination of PTEN is known to improve the activity of PI3/AKT pathway thus further proves its role as negative regulator of this pathway (Lee et al, 2013). Moreover, it was recently reported that TRIM27 is involved in the ubiquitination of PTEN in esophageal squamous cell carcinoma (Ma et al, 2019). Also it is known that the loss of PTEN is critical for stem cell maintenance and its loss is observed in CSC from variety of solid and hematological malignancies, where it may contribute to functional phenotype of CSCs (Ciuffreda et al, 2014). Besides this, PTEN is known to stabilize the concentrations of p53 by acetylation of p53 and its subsequent tetramerization. Moreover, p53 being tumor suppressor is known to supress the expression of self-renewal genes Sox2, Myc, Klf 4 and Oct 4.

Keeping these findings in view, we hypothesized that; TRIM27/PTEN/p53 axis may play a role in conferring stem cell characteristics to cancer cells in CRC. This incites us to design the model based on hypothesis where we predict the possible mechanism of action of TRIM27/PTEN/p53 axis in modulating the phenotype in cancer cells

towards CSC like phenotype. Thus, this work will elucidate the possible mechanism of action of TRIM27 via our predicted model and hence will contribute to our understanding of role of TRIM27 in conferring CSC phenotype and chemo-resistance in CRC. Moreover, the study will pave a way to identification of novel targets thus may contribute to the development of new therapeutic strategies. Finally, these strategies may result in the disruption of the machinery of stemness acquisition, which may prevent tumor growth, progression, and overcome the resistance to anticancer therapies.

2. Aim

To decipher the molecular mechanism of TRIM27/PTEN/p53 axis in conferring stem cell characteristics in colorectal cancer.

3. Objectives

1) To evaluate the role and fate of TRIM27-PTEN in Colorectal Cancer cell stemness.

2) To elucidate the underlying molecular mechanism of TRIM27 in contributing stem cell phenotype in PTEN cell model system.

3) To design the possible therapeutic strategy targeting stem cell phenotype in Colorectal Cancer model system.

4. Review of Literature

Colorectal cancer (CRC) is the third most diagnosed cancer with contribution of 10.2% of all cases in the world. It is the third leading cause of cancer related deaths in the world (WHO). According to the recent report published in CA: A Cancer Journal for Clinicians the colorectal cancer is ranked third in terms of estimated new cases

and deaths in both males and females in USA (Siegel et al, 2022). As per the recent report of National Cancer Registry Programme 2021 the CRC is ranked third most common cancer in both males and females in India (Indian Council of Medical Research- National Centre for Disease Informatics and Research). The CRC comprised over a quarter of the cancers in the younger age groups 20 to 34 years in both sexes with chemotherapy the most typical treatment modality for many cancers regardless of the clinical extent of the disease at presentation as per this report. The prevalence of CRC has been dramatically growing at an alarming rate globally. There are estimated 1.93 million new CRC cases diagnosed and 0.94 million CRC caused deaths in 2020 worldwide (Dekker et al. 2019). Based on the previous year trends one of the recent reports has presented the global epidemiology of CRC in 2020 and projections for 2040, in top 10 countries with highest number of new cases and deaths in 2020 and projections for 2040 **(Figure 1 and 2)** (Xi and Xu et al. 2021).

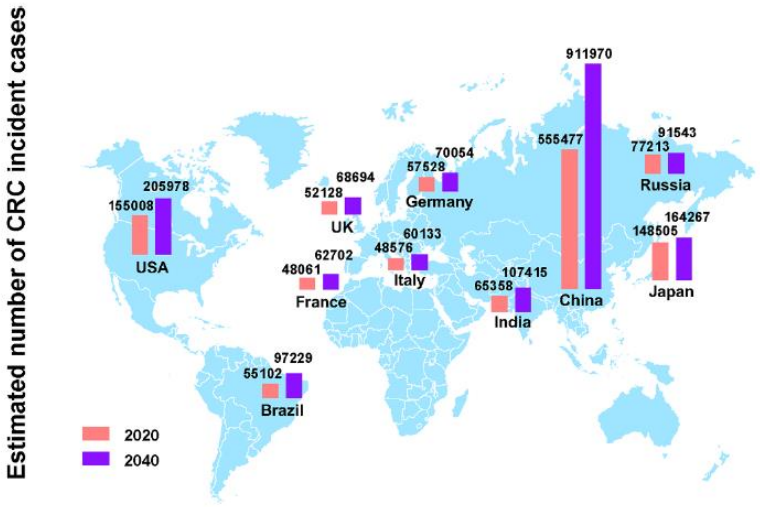

Figure 1: *The number of new CRC cases in top 10 countries with highest incident cases in 2020 and projections for 2040 (Xi and Xu et al. 2021).*

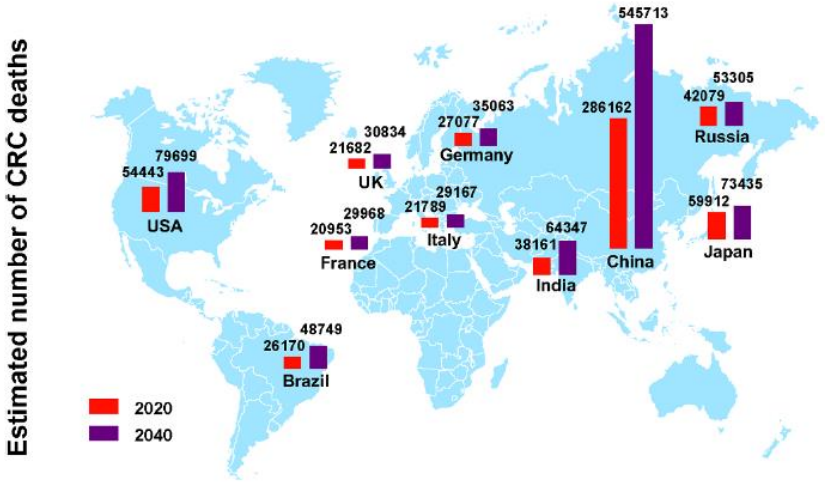

Figure 2: *Deaths from CRC cases in top 10 countries with highest incident cases in 2020 and projections for 2040 (Xi and Xu et al. 2021).*

Currently, therapeutic options for the treatment of CRC are surgical resection, systemic therapy including chemotherapy, targeted therapy and immunotherapy. Unfortunately even after well-directed curative treatment, patients experience treatment failure that may be associated with the development of multi-drug resistance during or after treatment. The CSCs are well-known in conferring the resistance to various therapeutic interventions. These are subpopulation of cells endowed with the capacity for self-renewal and tumor regenerating potential (Zhou et al, 2021). The inability to fully eradicate tumor-initiating CSC populations remains a significant hurdle in cancer therapy. CSCs have the ability to initiate various diseases, are often resistant to chemotherapy and radiation, and result in disease recurrence in cancer patients (Jordan et al. 2006; O'Brien et al, 2009; Kreso and Dick, 2014; Prager et al., 2020). Therefore, eradication of CSCs is likely to be an important component of improved cancer therapies. It has been postulated that effective targeting of CSCs

could result in deeper clinical remission, more durable responses, and potentially curative therapies (Pollyea and Jordan, 2017). CSCs have been reported in many tumor types including leukemia, breast cancer, bone cancer, brain cancers, colon cancer, prostate cancer, lung cancer, liver cancer and pancreatic cancer (Jones et al, 2021). Therefore, effectively targeting CSCs has the potential to improve patient outcomes across a broad range of cancer types. There are numerous mechanisms that enable cancer cells to acquire and maintain the stem cell phenotype. Most of them are interrelated and influence each other, considerably increasing the complexity of CSC regulation. There are several distinct mechanisms or biological processes that confer the unlimited self-renewal potential and the pluripotency to stem cells. Identification of growing number of TRIM proteins gained importance as crucial modifiers of stemness phenotype in various malignancies. An ever-growing number of studies demonstrate significant engagement of distinct TRIM family members in each of the abovementioned characteristics of CSCs.

The TRIM family of proteins represents one of the largest classes of putative single protein RING-finger E3 ubiquitin ligases. The members of this family are characterized by an N-terminal TRIM motif containing one RING-finger domain, one or two zinc-finger domains called B boxes (B1 box and B2 box), and a coiled-coil region. TRIM proteins regulate stem cell characteristics (mostly positively) by enhancing the activity of core transcription factors, induction of specific signaling pathways, epigenetic silencing of pro-differentiation genes, metabolic reprogramming, and activation of the EMT program. Several members negatively impact stem cell self-renewal presumably by ubiquitin-mediated degradation of stem cell transcription factors or inhibition of specific signaling pathways (Hatakeyama et al, 2017) **Figure 3** (Jaworska et al, 2019).

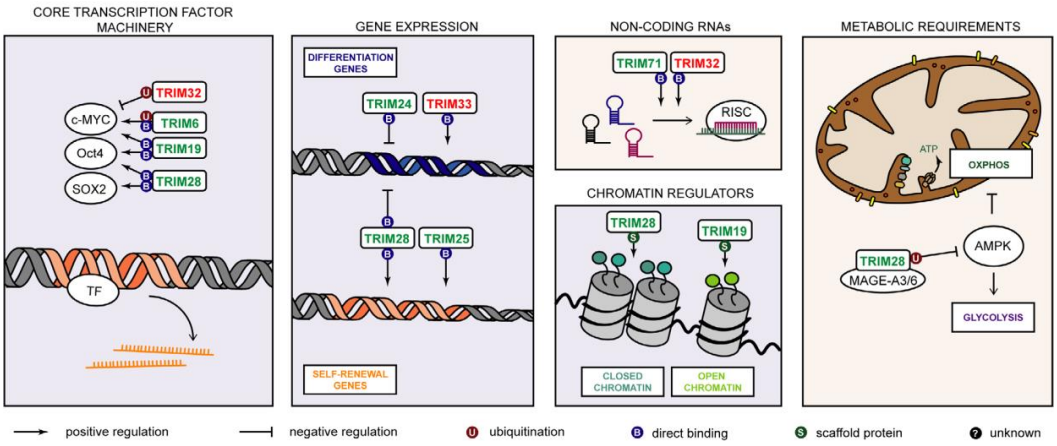

Figure 3*: Several distinct roles of TRIM family proteins in conferring the unlimited self-renewal potential and the pluripotency of stem cells. A growing number of TRIM proteins are being recognized as important modifiers of stemness machinery. Positive regulators of stem cell self-renewal are marked in green and negative regulators are marked in red. TRIM proteins are able to ubiquitinate (U, brown dot), directly bind target protein (B, dark blue dot), or serve as a scaffold (S, dark green dot) to exert their stem cell-associated function (Jaworska et al, 2019).*

TRIM27 an E3 Ubiquitin ligase is one of the family members of TRIM, and like other members inherits the basic structure of TRIM family (Micale et al, 2012). Previous report has demonstrated that TRIM27 potentiates the progression of CRC (Zhang et al, 2018). Moreover, it has also been confirmed that inhibiting the expression of TRIM27 suppresses the proliferation of ovarian (Ma et al, 2016) and nasopharyngeal cancer cells (Meng et al, 2017). In addition, TRIM27 positively regulates the TNF-α-induced apoptosis (Zaman et al, 2013). Importantly, it has been demonstrated that TRIM27 is involved in activation of PI3/AKT pathway in various cancers and is responsible for the ubiquitination of PTEN in esophageal squamous cell carcinoma (Ma et al, 2019). Researchers have also demonstrated that overexpression of TRIM27

promotes the proliferation of cancer cells in various malignancies like ovarian cancer (Ma et al, 2016) endometrial cancer (Tsukamoto et al, 2009) breast cancer (Tezel et al, 2009) lung cancer (Iwakoshi et al, 2012). Interestingly, it has been also demonstrated that TRIM27 promotes EMT and activates AKT signaling pathway in CRC (Zhang et al, 2018). One of the recent study have demonstrated that TRIM27 serves as a oncogene in non-triple negative breast cancer by targeting p21 for ubiquitination and its expression is related with the poor breast cancer prognosis (Xing et al, 2020). The overexpression of TRIM27 has been reported to drive the proliferation of cells in gastric cancer and it's down regulation supresses the proliferation via targeting and inhibiting Hippo-BIRC5 pathway (Yao et al, 2020). These studies demonstrate that TRIM27 functions as oncogene and can act as a positive regulator of stem cells in various cancers. However, the molecular mechanism of action of TRIM27 and its role in regulating CSCs in various cancers is poorly understood. So, elucidating the molecular modes of TRIM27 in governing CSC properties is necessary to optimize therapeutic approaches**.** Moreover, the E3 ubiquitin ligase activity mediated by the RING domain is commonly used by specific TRIM proteins to exert the stem cell-associated function. Besides the above mentioned targets for ubiquitination and proteasomal degradation, the level of several other proteins is tightly regulated by TRIM members to maintain the pluripotent state. These include the components of the cell cycle regulatory circuit or proteins associated with cell metabolism. Therefore, the presence of a catalytically active RING domain can serve as a therapeutic target that could be blocked by chemical compounds might make TRIM family members attractive targets for the manipulation of stem cell homeostasis in various malignancies.

5. Identification of Research Gaps

It is a well-established fact that CSCs play a critical role in tumor relapse, multi-drug resistance and metastasis. As a result they contribute to the shorter survival rate in CRC patients and remain a critical challenge in management of CRC. In order to improve the disease free survival of CRC patients, the understanding of the factors that contribute to CSC phenotype need to be studied. TRIM family of proteins are known to be involved in regulating an assortment of important biological processes, especially stem cell phenotype, proliferation and cellular differentiation. The plethora of studies has demonstrated TRIM27 one such member of TRIM family as an oncogene and its high expression in cancer cells is connected with cell cycle deregulation, tumour cell proliferation and migration. However, the role and mechanism of action of TRIM27 in regulating stem cell characteristics and associated multi-drug resistance remains elusive and has not been studied. In this work, understanding the role of TRIM27 in conferring stem cell characteristics to cancer cells and elucidating the related mechanism may possibly address the problems like multi-drug resistance in various malignancies especially CRC. This work will provide a deeper insight in understanding the molecular mode of action of TRIM27 and its role in governing CSC properties. Therefore, may prove necessary to optimize chemotherapeutic interventions for better clinical outcome and improved patient survival.

6. Methodology

Objective 1: To evaluate the role and fate of TRIM27-PTEN in colorectal cancer cell stemness.

Approach: The CRC cells (HCT116, LoVo, SW48, RKO and NCM460) will be screened for the expression of TRIM27, PTEN and p53. In order to investigate the role of TRIM27 in self-renewal and tumorigenesis the overexpression and knockdown studies will be carried in cells expressing low or high TRIM27 respectively. The effect of overexpression and knockdown of TRIM27 will be then investigated on the expression of self-renewal genes (Sox-2, Klf-4, c-Myc Oct-4) and CSC markers (CD133 and CD44). Moreover, the overexpression and knockdown of the TRIM27 will be also investigated in sphere formation assay of CRC cells.

•Cell culture

•Sphere formation assay

•Trim27, PTEN and p53 expression analysis in $CD133^+$ and $CD44^+$ colonspheres

•Generation of TRIM27 overexpression and knockdown cells

•Expression studies of self-renewal genes (Sox-2, Klf-4, c- Myc, Oct-4)

•Analysis of CSC markers (CD133 and CD44)

a) Cell culture: The selected cell lines (HCT116, LoVo, SW48, and RKO) will be cultured in DMEM medium and 10% fetal bovine serum albumin (FBS) supplemented with 1% penicillin/streptomycin and 2mM L-glutamine.

b) Sphere formation assay: The cells will be originally cultivated in PCM (Prefusion medium), trypsinized and maintained in serum-free stem cell medium containing Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with ITS Liquid Media Complement, 5 mM HEPEs, 4 mg/ml BSA, 2 nM L-glutamine, 3 mg/ml glucose, 20 ng/ml EGF, 20 ng/ml βFGF.

c) Trim27, PTEN and p53 expression analysis in $CD133^+$ and $CD44^+$ colonspheres: The expression of TRIM27, PTEN and p53 will be checked in the cells/spheres using western blot using particular TRIM27, PTEN and p53 antibodies.

d) Generation of TRIM27 overexpression and knockdown cells: The overexpression studies of the TRIM27 gene will be carried by cloning it in lentiviral vector pLKV-Puro by standard cloning techniques. For transient cell line generation the cells HEK293T will be transfected with a pLKV-Puro expression vector. Following the supernatant harvesting containing lentivirus the stable CRC SW48 and RKO cell line will be generated. After incubation of the supernatant with SW48 and RKO cells the positive clones will be selected and expanded for further experiments. For knockdown studies the silencing of TRIM27 with either siRNA or shRNA will be carried in LoVo and HCT116 cells. These TRIM27 targeting siRNA/shRNA will be transfected into cells by Lipofectamine 2000. The nonspecific scrambled siRNA or shRNA sequence will serve as negative control. The western blot analysis will be carried for confirming the overexpression and knockdown studies of TRIM27 in the transfected cells using anti-TRIM27 antibodies.

e) Expression studies of self-renewal genes (Sox-2, Klf-4, Oct-4 and Myc): The percentage of cells expressing the CSC markers CD133/CD44 will be analyzed by flow cytometry after overexpression and knockdown of TRIM27. Further to analyze the expression of stem-related genes including Sox2, Myc, Klf 4 and Oct 4 will be investigated in these cells by qRT- PCR using gene specific primers.

Objective 2: To elucidate the underlying molecular mechanism of TRIM27 in contributing stem cell phenotype in PTEN cell model system.

Approach: The interaction of TRIM27 as a result ubiquitination of PTEN will be studied in order to understand the role of TRIM27-PTEN in conferring stemness to the cancer cells. The effect of PTEN knockdown on expression of self-renewal genes (Sox-2, Klf-4, c- Myc, Oct-4) will be investigated to ascertain the role of TRIM27-PTEN axis on stemness. Then together the effect of p53 inhibition in PTEN knockdown cells on the expression of self-renewal genes (Sox-2, Klf-4, c- Myc, Oct-4) and its role in regulating the expression of these genes at promoters will be further studied. This will ascertain the role of TRIM27/PTEN/p53 axis in conferring the stem cell phenotype in CRC cells.

- Interaction studies between TRIM27 and PTEN
- Effect of PTEN knockdown on self-renewal genes (Sox-2, Klf-4, c- Myc, Oct-4) and cell proliferation
- p53 inhibitor treatment and its effect on self-renewal in PTEN knockdown cells (Sox-2, Klf-4, c- Myc, Oct-4)
- p53 binding analysis to promoters of self-renewal genes (Sox-2, Klf-4, c-Myc, Oct-4)

a) Interaction studies between TRIM27 and PTEN: The co-immunoprecipitation studies will be done in order to check this interaction in CRC. Here the interaction will be seen in both TRIM27 overexpressing (SW48 and RKO) and knockdown cells (LoVo and HCT116). The whole cell lysates will be incubated with anti-TRIM27, anti-PTEN and normal IgG antibodies with protein A/G PLUS Agarose beads for 2 hour at 4°C. The beads after proper washing will be separated by SDS-PAGE followed by western blot analysis. Moreover the ubiquitination assay will be done to

study ubiquitination of PTEN by TRIM27. The endogenous PTEN here will be precipitated by anti-PTEN antibody and ubiquitination on PTEN will be confirmed by western blotting using anti-ubiquitin antibody. Further, the PTEN protein levels will be investigated in TRIM27 overexpressing and knockdown cells by western blotting.

b) Effect of PTEN knockdown on self-renewal genes (Sox-2, Klf-4, Oct-4 and Myc): To understand the role of PTEN as a substrate in mediating the effect of TRIM27 on CSC phenotype in CRC cells, the knockdown of PTEN expression by either shRNA/siRNA in TRIM27 deficient cells LoVo and HCT116 will be done. The expression analysis of stem-related genes (Sox2, Myc, Klf 4 and Oct 4) will be achieved by qRT- PCR. The protein levels of these factors will be analyzed by western blotting in these cells.

c) p53 inhibitor treatment and its effect on self-renewal genes (Sox-2, Klf-4, Oct-4 and Myc): The role of p53 as a tumor suppressor is involved in preventing the transition of cancer cells to CSCs, thus plays role in suppression of stemness. Here the effect of p53 inhibition on expression of stem-related genes (Sox2, Myc, Klf 4 and Oct 4) will be analyzed by examining RNA levels by qRT- PCR. The cells TRIM27 and PTEN knockdown LoVo and HCT116 will be used for this experiment.

d) p53 binding analysis to promoters of self-renewal genes (Sox-2, Klf-4, Oct-4 and Myc): The chromatin immunoprecipitation and PCR assays will be performed in order to check the p53 binding to promoters of self-renewal genes. This binding assay will be investigated in TRIM27 overexpressing (SW48 and RKO cells) and knockdown cells (LoVo and HCT116 cells) along with cells that are attenuated for the expression of both TRIM27 and PTEN.

Objective 3: To design the possible therapeutic strategy targeting stem cell phenotype in Colorectal Cancer model system.

Approach: In order to understand and validate the role of TRIM27-PTEN axis in more realistic model the xenograft mouse model will be used. Further, human CRC specimens will be studied and evaluated for clinical significance and possible new therapeutic interventions.

- Xenograft mouse model generation
- Expression analysis of CSC surface markers and self-renewal genes
- Expression analysis of TRIM27, PTEN, p53 and CSC markers in human specimens
- Evaluation of clinical significance

a) Xenograft mouse model generation: The six week old nude mice will be subcutaneously injected with LoVo cells stably transduced with shRNA at their right armpit. The other group will be injected with SW48 stably transduced with TRIM27 at their right armpit.

b) Expression analysis of CSC surface markers and self-renewal genes: The mice will be sacrificed at 4 weeks post injection, and tumor tissues will be surgically removed to analyse tumor growth. The samples will be analysed for the expression of PTEN, p53 and self-renewal genes Sox-2, Klf-4, c-Myc, Oct-4 by western blotting and qRT-PCR respectively.

c) Expression analysis of TRIM27, PTEN, p53 and CSC markers in human specimens: The CRC tissue specimens from patients will be investigated for the

expression of TRIM27, PTEN, p53 and CSC markers (CD133 and CD44) by IHC staining.

d) Evaluation of clinical significance: The correlation between the expression of TRIM27 and clinical parameters of patients. The clinical parameters to be studied will be tumor size, tumor stage, distant metastasis and survival time.

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